Multiple substitutions at position 104 of β-lactamase TEM-1: assessing the role of this residue in substrate specificity

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Residue 104 is frequently mutated from a glutamic acid to a lysine in the extended-spectrum TEM β-lactamases responsible for the resistance to third-generation cephalosporins in clinical Gram negative strains. Among class A β-lactamases, it is the most variable residue within a highly conserved loop which delineates one side of the active site of the enzymes. To investigate the role of this residue in the extended-spectrum phenotype, it has been replaced by serine, threonine, lysine, arginine, tyrosine and proline. All these substitutions yield active enzymes, with no drastic changes in kinetic properties compared with the wild-type enzyme, except with cefaclor, but an overall improved affinity for second- and third-generation cephalosporins. Only mutant E104K exhibits a significant ability to hydrolyse cefotaxime. Molecular modelling shows that the substitutions have generally no impact on the conformation of the 101–111 loop as the side chains of residues at position 104 are all turned towards the solvent. Unexpectedly, the E104P mutant turns out to be the most efficient enzyme. All our results argue in favour of an indirect role for this residue 104 in the substrate specificity of the class A β-lactamases. This residue contributes to the precise positioning of residues 130–132 which are involved in substrate binding and catalysis. Changing residue 104 could also modify slightly the local electrostatic potential in this part of the active site. The limited kinetic impact of the mutations at this position have to be analysed in the context of the microbiological problem of resistance to third-generation cephalosporins. Although mutation E104K improves the ability of the enzyme to hydrolyse these compounds, it is not sufficient to confer true resistance, and is always found in clinical isolates associated with at least one mutation at another part of the active site. It is the combined effect of the two mutations that synergistically enhances the hydrolytic capability of the enzyme towards third-generation cephalosporins.

INTRODUCTION

Class A β-lactamases are a major therapeutic problem in treating bacterial infections with the widely used β-lactam antibiotics (Bush, 1989; Ambler et al., 1991). In the 1980s, a new generation of broad spectrum cephalosporins such as cefotaxime and ceftazidime, resistant to hydrolysis by β-lactamases, was introduced. Clinical use of these so-called third-generation cephalosporins led to the selection of new, extended-spectrum β-lactamases among enterobacteria, as early as 1983.

Over 30 such enzymes have now been found worldwide and interestingly, under similar antibiotherapeutic selective pressure, the same mutant enzymes arose independently in several places (Jacoby and Medeiros, 1991; Chanal et al., 1992; Naumovski et al., 1992; Vedel et al., 1992). A total of 23 such extended-spectrum enzymes have been shown to be derivatives of the TEM enzyme. Indeed, these enzymes differ from TEM-1 by a limited number of mutations (usually two to four) mainly affecting residues within or next to the active site. Among these mutations, a change of glutamic acid 104 to lysine is frequently encountered. Previous work by us and others has shown that this mutation alone is partly responsible for the extended substrate range of the new TEM enzymes (Sougakoff et al., 1989; Lenfant et al., 1990; Sowek et al., 1991). In the absence of precise structural data on the TEM enzyme and the enzyme–substrate complex, it was proposed that such a mutation would favour hydrolysis of the oxyimino-cephalosporins by promoting new electrostatic interactions between the *N*-oxyimino group of the substrate and the active site of the enzyme.

Sequence alignments show that the region around residue 104 is one of the most conserved among class A β-lactamases (Table 1). This is all the more true for the enzymes from Gram negative bacteria. Curiously, residues 101 to 111 are essentially conserved, except for residue 104. In fact, only TEM-1 and the *Nocardia lactamdurans* enzyme (along with some of the natural TEM mutants) bear a glutamic acid at this position among the class A enzymes.

Analysis of the high-resolution three-dimensional structure of the TEM-1 enzyme (Jelsch et al., 1993) shows that residue 104 is within a large loop encompassing residues 101 to 111 (Figure 1). This loop is stabilized by a network of hydrogen bonds with the N-terminal part of helix H5 (residues 132–142) and water molecule 459, including several hydrogen bonds involving the main-chain atoms of glutamic acid 104 (Figure 2). Water molecule 459 also interacts with the carboxylate of the side chain of glutamic acid 104, which is totally exposed to solvent. In the crystal structure, glutamic acid 104 is 10 Å from the active site serine-70 hydroxyl, involved in the acylation reaction which is the first step in β-lactam hydrolysis. It is 6.9 Å from the carboxylate of glutamic acid 166 (involved in the deacylation step) and 10.4 Å from lysine 73 which, according to Strynadka et al. (1992), acts as the general base in the acylation step. Therefore, glutamic acid 104 does not interact directly with these active-site residues. However, the main-chain oxygen of glutamic acid 104

Abbreviation used: MIC, minimal inhibitory concentration.

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Table 1 Sequence alignment for the 101–111 loop of some class A β-lactamases

Numbering of amino acid residues is according to Ambler et al. (1991). The sequences are from Ambler et al. (1991) except for TEM-3, TEM-6, TEM-7 and TEM-9 (Jacoby and Medeiros, 1991); *Proteus mirabilis* GN79 (Sakurai et al., 1991); *Klebsiella oxytoca* D488 (Reynaud et al., 1991); MEN-1 (Barthélémy et al. 1992); *Proteus vulgaris* RO104 (EMBL P80298); ROB-1 (Livrelli et al., 1991); *Nocardia lactamdurans* (NBRF S222750).

	Residue										
Enzyme	101	102	103	104	105	106	107	108	109	110	111
TEM-1	**D**	**L**	**V**	**E**	**Y**	**S**	**P**	**V**	**T**	**E**	**K**
TEM-3	**D**	**L**	**V**	K	**Y**	**S**	**P**	**V**	**T**	**E**	**K**
TEM-6	**D**	**L**	**V**	K	**Y**	**S**	**P**	**V**	**T**	**E**	**L**
TEM-7	**D**	**L**	**V**	E	**Y**	**S**	**P**	**V**	**T**	**E**	**K**
TEM-9	**D**	**L**	**V**	K	**Y**	**S**	**P**	**V**	**T**	**E**	**K**
SHV-2	**D**	**L**	**V**	D	**Y**	**S**	**P**	**V**	S	**E**	**K**
LEN-1	**D**	**L**	**V**	D	**Y**	**S**	**P**	**V**	S	**E**	**K**
PSE-4	**D**	**L**	**V**	T	**Y**	**S**	**P**	**V**	I	**E**	**K**
PSE-3	A	**L**	**V**	T	**Y**	**S**	**P**	**V**	**T**	**E**	R
P. mirabilis GN79	N	**L**	**V**	T	**Y**	**S**	**P**	**V**	**T**	**E**	**K**
K. oxytoca D488	**D**	**L**	**V**	V	W	**S**	**P**	I	**T**	**E**	**K**
MEN-1	**D**	**L**	**V**	N	**Y**	N	**P**	I	A	**E**	**K**
P. vulgaris RO104	**D**	**L**	**V**	A	**Y**	**S**	**P**	I	**T**	**E**	**K**
ROB-1	**D**	**L**	**V**	S	**Y**	**S**	**P**	E	**T**	Q	**K**
B. licheniformis	**D**	**L**	**V**	N	**Y**	N	**P**	I	**T**	**E**	**K**
B. cereus III	**D**	**L**	**S**	N	**Y**	N	**P**	I	**T**	**E**	**K**
B. cereus 569/H	**D**	**L**	**V**	D	**Y**	**S**	**P**	**V**	**T**	**E**	**K**
S. aureus PC1	**D**	**L**	**V**	A	**Y**	**S**	**P**	I	L	**E**	**K**
N. lactamadurans	E	**L**	L	E	N	**S**	**P**	I	**T**	K	D
S. cacaoi blaU	**D**	**L**	**V**	D	N	**S**	**P**	**V**	**T**	**E**	**K**
S. basius	**D**	**L**	**V**	A	H	**S**	**P**	**V**	**T**	**E**	**K**
S. cacaoi ULg	A	I	L	P	N	**S**	**P**	**V**	**T**	**E**	**K**
Consensus	**D**	**L**	**V**	X	**Y**	**S**	**P**	**V**	**T**	**E**	**K**

makes a hydrogen bond with the amide group of aspargine 132 which has been shown by Strynadka et al. (1992) to interact directly with the substrate.

In the light of this new body of structural data, it was interesting to try to reassess the role of residue 104 in the substrate specificity of the TEM β-lactamases. For this purpose, several mutants were constructed by site-directed mutagenesis, and computer modelling of the enzyme–substrate complexes was performed to try to rationalize the kinetic data obtained.

MATERIALS AND METHODS

Media, enzymes and chemicals

Bacterial cells were grown at 30 °C in Luria–Bertani medium (Difco Laboratories, OSI, Marepas, France) supplemented with the appropriate antibiotics: 100 mg/l ampicillin (Bristol Laboratory, Paris La Défense, France), 30 mg/l chloramphenicol (Serva, Saint-Germain-en-Laye, France) and 12 mg/l tetracycline (Boehringer Mannheim). For enzyme preparations, cells were grown at 37 °C in Trypticase Soya broth (Difco) supplemented with 12 mg/l tetracycline. Bacterial susceptibility to β-lactams was measured at 37 °C on Mueller–Hinton agar (Diagnostics Pasteur, Marne-la-Coquette, France). Minimal inhibitory concentration (MIC) determinations were performed by agar dilution with an inoculum of 10^4 colony-forming units/spot. [α-^{35}S]dCTP was purchased from Amersham (Les Ulis, France). The helper phage M13K07, enzymes for DNA technology and T7 polymerase sequencing kit were purchased from Pharmacia LKB Biotechnology (Saint-Quentin en Yvelines, France). The β-lactam powders were kindly provided by the following laboratories: amoxicillin, carbenicillin, clavulanic acid and ticarcillin, Beecham-Sevigné (Nanterre, France); penicillin G, Specia; piperacillin, Lederle (Oullins, France); cephalothin, cefaclor and latamoxef, Lilly (Saint-Cloud, France); cefaloridine and ceftazidime, Glaxo (Paris, France); cefoperazone, Pfizer (Orsay, France); cefotaxime, Roussel–Uclaf (Romainville, France); aztreonam, Squibb (Paris la Défense, France). Structures of the compounds are given in Figure 3.

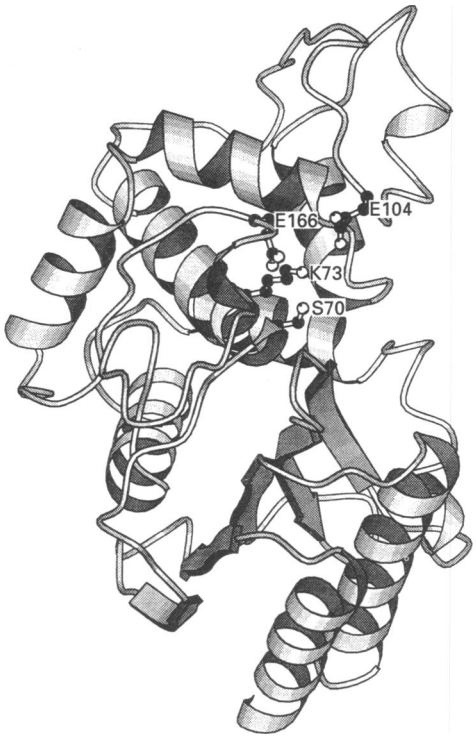

Figure 1 Position of residue 104 in the TEM-1 structure

The main catalytic residues, serine 70, glutamic acid 166 and lysine 73 are also shown.

Bacterial strains and plasmids

Bacterial strains *Escherichia coli* XAC-1, plasmids pCT1 and pCT3 have been described previously (Lenfant et al., 1990). Plasmid DNA purification and cloning techniques were based on Sambrook et al. (1989). The preparation of competent *E. coli* cells and subsequent transformation with plasmid DNA were carried out according to Hanahan (1965).

Oligonucleotides and site-directed mutagenesis

Synthetic oligonucleotides were made and phosphorylated as previously described (Lenfant et al., 1990). The following oligonucleotides were used: E104am: 5′GTGAGTACTAAACCAAGTC3′; E104K: 5′GTGAGTACTTAACCAAGTC3′; E104R: 5′GTGAGTACCTAACCAAGTC3′; E104S: 5′GTGAGTACGAAACCAAGTC3′; E104T: 5′GTGAGTACGTAACCAAGTC3′; E104P: 5′GTGAGTAGGGAACCAAGTC3′; E104Y: 5′GTGAGTAATAAACCAAGTC3′.

Site-directed mutagenesis was performed using Eckstein's method (Taylor et al., 1985). The E104 amber was selected for its sensitivity to ampicillin. Missense revertants were obtained by the same method starting from the amber-mutated gene. The mutations were confirmed by sequencing using the dideoxy method (Sanger et al., 1977).

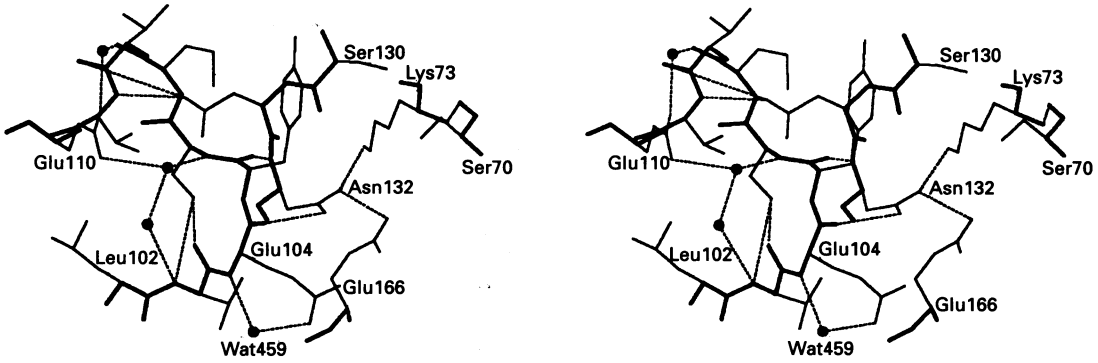

Figure 2 Stereo view of the 101–111 loop and the active site of TEM-1

Heavy lines, main-chain atoms; narrow lines, side chains; broken lines, hydrogen bonds; ●, water molecules

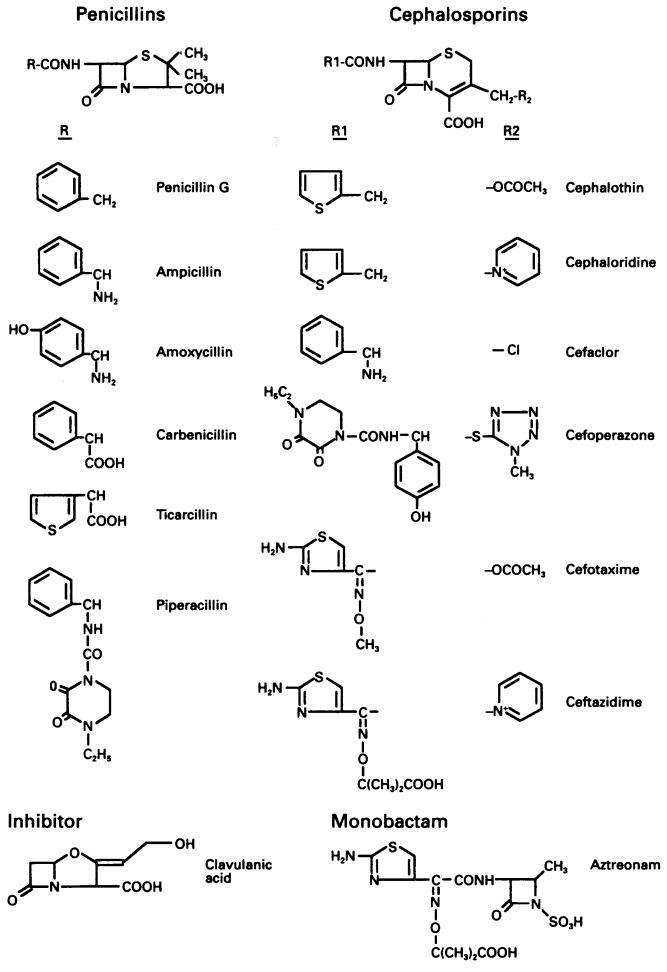

Figure 3 Structure of the β-lactam antibiotics used in this study

β-Lactamase purification

Cells from 10 litre cultures were collected during exponential-growth phase by centrifugation at 5000 *g* for 10 min, washed with distilled water, resuspended in 5 g/l NaCl, broken by sonication and centrifuged at 1000 *g* for 30 min. The wild-type and protein variants were purified as described (Delaire et al., 1992) The β-lactamase activity was detected with a spectrophotometer at 30 °C in 50 mM sodium phosphate buffer, pH 7, with cephaloridine as substrate ($\lambda = 260, \Delta\epsilon = 10200\ M^{-1} \cdot cm^{-1}$). Protein concentration was determined by absorbance measurements at 280 nm with $\epsilon = 18200\ M^{-1} \cdot cm^{-1}$. The β-lactamase

preparations were then found to be homogeneous as judged by analytical SDS-phastgel (Pharmacia).

Kinetic parameters

The kinetic constants were determined at 37 °C and pH 7 by computerized microacidometry (Labia et al., 1973). The inhibition constant K_i has been measured using competition procedures with penicillin G for compounds for which no or only weak hydrolysis could be detected. For clavulanic acid, the rate of substrate hydrolysis decreases with time and kinetics can be fitted to the following reaction mechanism:

$$E + I \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} (E, I) \xrightarrow{k_{+2}} (EI) \underset{k_{+3}}{\overset{k_{+4}}{\rightleftharpoons}} \begin{matrix} E - I \\ E + P \end{matrix}$$

Inhibition by clavulanic acid was tested by preincubation with the enzyme at 37 °C for 10 min before the rate of hydrolysis of penicillin G was measured. The I_{50} values were obtained graphically as the concentrations of inhibitor causing 50 % inhibition of benzylpenicillin hydrolysis after incubation of the enzymes at 37 °C for 10 min with the inhibitor. A turnover number ($t_n = k_{+3}/k_{+4}$) was deduced from the extrapolated value for 100 % inhibition. The value for $k_{inac.}$, the irreversible inactivation rate constant, has been calculated from the half-life of the enzyme, $t_{\frac{1}{2}}(k_{inac.} = \ln 2/t_{\frac{1}{2}}; k_{hydrol.} = k_{inac.} \cdot t_n)$. Catalytic efficiency is defined as $k_{cat.}/K_m$ for substrate hydrolysis or k_{hydrol}/K_i for inhibitor hydrolysis. An inhibition efficiency for irreversible inhibitors can be defined in the same manner as $k_{inac.}/K_i$. All the kinetic parameters are determined in at least four independent experiments. One unit of β-lactamase activity is defined as the amount of enzyme hydrolysing 1 μmol of penicillin G/min at pH 7 and 37 °C.

Isoelectric points (pI) were determined by analytical isoelectric focusing revealed by the iodine procedure in agar gel with penicillin G as substrate (Labia and Barthélémy, 1979).

Modelling experiments

Modelling of the amino acid substitutions was made from the refined X-ray structure of the wild-type enzyme (Jelsch et al., 1993) with the molecular graphics program FRODO (Jones, 1978). Replacement of the solvent-exposed glutamic acid was straightforward; the side-chain conformations were kept at their minimum conformational energy. In the case of proline, the main-chain constraints were accommodated through minor changes of the (Φ, Ψ) dihedral angles of residues 103 and 104.

RESULTS

Susceptibility of the *E. coli* strains producing the mutant enzymes

In a previous work Lenfant et al. (1990) have shown by information suppression that all substitutions tested at position 104 resulted in active β-lactamases. To perform more thorough studies, the amber codon 104 was reverted by oligonucleotide-directed mutagenesis to the codons AAG, AGG, UCG, ACG, GGG and UAU, and the mutant genes were expressed in plasmid pCT3, yielding the β-lactamase variants E104K, E104R, E104S, E104T, E104P and E104Y.

Table 2 shows the levels of resistance to β-lactams of the host strain and of the strains producing the TEM enzymes. Strains harbouring the mutant genes are highly resistant to amoxycillin and ticarcillin, as for TEM-1, and clavulanic acid decreases the MIC to amoxycillin, but to a different extent. They are moderately resistant to cephalothin and exhibit a full or reduced susceptibility to the other compounds. Only XAC-1 E104K is less susceptible to cefotaxime than the wild-type harbouring strain. Overall, strains E104S and E104P are more resistant than TEM-1, but the increase is not spectacular. Among the other mutants, E104R performs rather badly, and so does E104Y.

These MIC values show that all the mutant enzymes are active, but they do not seem to modify the resistance pattern of their host strain in a drastic way. The pI of the mutant enzymes E104K and E104R are identical and close to 5.9 whereas the other mutant enzymes have a pI close to 5.5. The pI value for TEM-1 is 5.4.

Kinetics

The k_{cat} values for penicillins vary in a narrow range compared with the wild-type values (Table 3). While E104T has roughly 50 % of the wild-type activity, E104P, E104S and E104Y are clearly more active than the wild-type, but the only outstanding values are for penicillin hydrolysis by E104Y. For cephalosporins, E104S, E104Y and E104P exhibit higher k_{cat} values than TEM-1 for all these substrates. The only outstanding features are the relatively higher activity of all the mutants towards cefaclor and the real ability of E104K to hydrolyse cefotaxime.

In terms of K_m, there are again no large variations for penicillins (Table 4). E104T and E104P have lower K_m values for all penicillins than the wild-type, but within a 2-fold range, while the other mutants have K_m values varying between 60 and 170 % of those of the wild-type. For cephalosporins, the situation is slightly more complicated. There is a sharp increase of K_m with cefaclor for all mutants, while these values are all decreased for cefoperazone. For the other third-generation cephalosporins, the K_m values of all the mutants are lower than the wild-type values, except for E104K in the case of cefotaxime. Variations for aztreonam are not significant, due to the rather large error (40 %) on the measurement of its K_i.

These variations of k_{cat} and K_m result in catalytic efficiencies (Table 5) which are 3- to 10-fold lower for the mutants with cefaclor, compared with wild-type TEM-1, while they vary in the opposite direction for cefoperazone. Among the mutants, only E104R and E104T are overall less active than the wild-type. Mutants E104S and E104Y are slightly better enzymes than TEM-1, and E104P is clearly more efficient, both towards penicillins and cephalosporins.

The increased enzymatic efficiencies toward third-generation cephalosporins of several mutants have various origins. In fact, E104K is the only mutant which has significantly increased its hydrolytic rate for cefotaxime, over a threshold that actually translates into a biologically significant increase in MIC.

Interactions with clavulanic acid

Inhibition by the suicide inhibitor clavulanic acid is also assayed for the various mutants. This compound reacts with the active-site residues in a stepwise mechanism (Imtiaz et al., 1993). The first step involves the formation of an acyl enzyme, as with all the substrates, but the hydrolysis of this reaction intermediate either leads to expulsion of the product out of the active site or formation of a covalent adduct with yet unknown residues of the active site, resulting in inactivation of the enzyme. Whatever its precise mode of action, clavulanic acid is very finely tuned to the

Table 2 MIC values for *E. coli Xac*-1 and the strains harbouring TEM-1 or its mutants

The antibiotics used were: AMX, amoxycillin; CA, clavulanic acid (2 μg/ml); TIC, ticarcillin; CF, cephalothin; CTX, cefotaxime; CAZ, ceftazidime; ATM, aztreonam; MOX, latamoxef. MICs are expressed in μg/ml.

	AMX	AMX + CA	TIC	CF	CTX	CAZ	ATM	MOX
Xac-1	8	8	4	8	0.06	0.12	< 0.007	0.06
TEM-1	> 1024	64	> 1024	32	0.12	0.25	0.015	0.12
E104T	> 1024	64	> 1024	32	0.06	0.50	0.015	0.12
E104S	> 1025	256	> 1024	128	0.12	0.50	0.015	0.12
E104K	> 1024	128	> 1024	64	0.25	1.00	< 0.007	0.12
E104R	> 1024	32	512	16	0.06	0.25	< 0.007	0.12
E104Y	512	16	512	64	0.12	0.25	< 0.007	0.12
E104P	> 1024	256	> 1024	128	0.06	0.50	0.030	0.12

Table 3 k_{cat} (s^{-1}) for TEM-1 and its mutants

S.D. ⩽ 20%.

	TEM-1	E104T	E104S	E104K	E104R	E104Y	E104P
Penicillin G	1100	650	1500	840	650	830	1500
Ampicillin	1200	480	1300	750	710	2200	1500
Amoxycillin	1000	460	1200	630	550	1280	1200
Ticarcillin	110	75	130	120	140	250	140
Carbenicillin	110	80	130	120	110	250	130
Piperacillin	1000	380	1000	590	650	830	1500
Cephalothin	150	85	600	150	90	250	650
Cefaloridine	1000	500	1100	670	750	1400	3000
Cefaclor	80	50	120	110	85	120	290
Cefoperazone	460	250	600	330	300	580	870
Cefotaxime	2	3	9	25	4	3	7
Ceftazidime	0.02	0.05	0.1	0.3	0.2	0.04	0.3
Aztreonam	0.2	0.5	1	2.5	3	0.8	1

Table 4 K_m (μM) for TEM-1 and its mutants

For K_m values lower than 300 μM, S.D. ⩽ 10%; for values higher than 300 μM, S.D. ⩽ 20%.

	TEM-1	E104T	E104S	E104K	E104R	E104Y	E104P
Penicillin G	24	21	31	21	17	20	17
Ampicillin	35	32	38	58	34	60	28
Amoxycillin	43	32	27	37	30	50	24
Ticarcillin	14	09	09	08	09	23	06
Carbenicillin	14	13	15	09	19	20	06
Piperacillin	43	24	30	34	30	28	40
Cephalothin	260	280	660	240	200	260	300
Cephaloridine	370	360	660	600	410	470	590
Cefaclor	50	370	640	400	600	230	510
Cefoperazone	240	70	80	90	70	70	80
Cefotaxime	1100*	380*	320*	1000	310*	310*	130*
Ceftazidime	300*	130*	250*	80*	230*	140*	170*
Aztreonam	200*	340*	210*	160*	200*	290*	180*

* Measured as K_i by competition with penicillin G.

active-site topology, thus providing a simple probe to assess topological variations. The kinetic data obtained with this inhibitor are consistent with those obtained with the substrates (Table 6).

The inhibition constant K_i does not vary by more than 40%, and neither does the inactivation constant $k_{inac.}$ (except for E104S) but the t_n values, which reflect the ratio between turnover of the compound and inhibition, vary much more widely. Mutant

Table 5 Catalytic efficiency, k_{cat}/K_m ($M^{-1} \cdot s^{-1}$), for TEM-1 and its mutants

	TEM-1	E104T	E104S	E104K	E104R	E104Y	E104P
Penicillin G	4.5×10^7	2.6×10^7	4.8×10^7	4×10^7	3.8×10^7	4.1×10^7	8.8×10^7
Ampicillin	3.4×10^7	1.5×10^7	3.4×10^7	1.3×10^7	2.1×10^7	3.7×10^7	5.4×10^7
Amoxycillin	2.3×10^7	1.4×10^7	4.4×10^7	1.7×10^7	1.8×10^7	2.6×10^7	5.0×10^7
Ticarcillin	8.0×10^6	8.0×10^6	1.4×10^7	1.5×10^7	1.5×10^7	1.1×10^7	2.3×10^7
Carbenicillin	8.0×10^6	6.0×10^6	9.0×10^6	1.3×10^7	6.0×10^6	1.2×10^7	2.2×10^7
Piperacillin	2.3×10^7	1.6×10^7	3.3×10^7	1.7×10^7	2.2×10^7	3×10^7	3.7×10^7
Cephalothin	6.0×10^5	3.0×10^5	9.0×10^5	6.2×10^5	4.5×10^5	9.6×10^5	2.2×10^5
Cefaloridine	2.7×10^6	1.4×10^6	1.7×10^6	1.1×10^6	1.8×10^6	3×10^6	5.1×10^6
Cefaclor	1.6×10^6	1.3×10^5	1.9×10^5	2.7×10^5	1.4×10^5	5.2×10^5	5.7×10^5
Cefoperazone	1.9×10^6	3.6×10^6	7.5×10^6	3.7×10^6	4.3×10^6	8.3×10^6	1.1×10^7
Cefotaxime	1.8×10^3	8×10^3	2.8×10^4	2.5×10^4	1.3×10^4	1×10^4	5.4×10^4
Ceftazidime	66	380	400	3.7×10^3	870	290	180
Aztreonam	1×10^3	1.5×10^3	4.8×10^3	1.6×10^4	1.5×10^4	2.8×10^3	5.5×10^3

Table 6 Kinetic parameters for wild-type TEM-1 and its mutants with clavulanic acid

Cat. eff. = catalytic efficiency; inh. eff = inhibition efficiency.

	K_i (μM)	I_{50} (μg/ml)	k_3/k_4	$k_{inac.}$ (s^{-1})	h_{hydrol} (s^{-1})	Cat. eff ($M^{-1} \cdot s^{-1}$)	Inh. eff. ($M^{-1} \cdot s^{-1}$)
TEM-1	0.10	0.020	130	0.04	5.2	52×10^6	4.0×10^5
E104T	0.07	0.009	20	0.03	0.6	8.60×10^6	4.3×10^5
E104S	0.08	0.045	325	0.07	22.8	285×10^6	8.8×10^5
E104K	0.10	0.021	85	0.05	4.3	43×10^6	5.0×10^5
E104R	0.08	0.012	30	0.03	0.9	11.3×10^6	3.8×10^5
E104Y	0.14	0.006	25	0.04	1.0	7.1×10^6	2.8×10^5
E104P	0.11	0.027	260	0.05	13.0	118×10^6	4.5×10^5

enzymes that are clearly more active against penicillins like E104P and E104S also hydrolyse clavulanic acid more efficiently and thus end up being more resistant to the antibiotic than TEM-1. However, E104R and E104Y hydrolyse it less efficiently and are therefore more sensitive. For these mutants, the kinetic data correlate well with the MIC values (Table 2, AMX+CA).

For the other mutants, the situation is less clearcut. The enzyme E104K behaves almost like the wild-type, except for a moderate decrease in t_n. The MIC value for the strain bearing this mutant is nonetheless better than for the wild-type. Mutant enzyme E104T is more sensitive to the inhibitor, due to a sharp drop in the t_n value, but this does not translate into an increased sensitivity of the strain in terms of MIC value.

DISCUSSION

The 101–111 loop is strongly conserved among all β-lactamases, although no obvious functional reason for this conservation can be found. Within this loop, only residue 104 is highly variable. While such loops are generally perceived as flexible, this particular loop is made rigid by a number of hydrogen bonds (Figure 2) within the loop and with the N-terminal part of helix H5. It is also facing another rigid loop that plays a major role in catalysis, the Ω loop bearing glutamic acid 166 (Figure 1). Among all the known class A β-lactamases sequences, only TEM-1 and the *N. lactamadurans* enzyme (Gram positive) have a glutamic acid at this position. In extended-spectrum enzymes TEM-3, TEM-4, TEM-6 and TEM-9, it is replaced by a lysine (Table 1).

Several authors hypothesized that extended-spectrum β-lactamases owed their new hydrolysing capabilities to the establishment of new hydrogen bonds or salt bridges between the substrate and the mutated residues, in particular lysine 104. This assumption was based on purely chemical rationale (Collatz et al., 1989; Sougakoff et al., 1989) or interpretation of a structural model from an enzyme 45% homologous to TEM-1 (Sowek et al., 1991). We now have several lines of argument against this hypothesis. As residue 104 is turned towards the solvent, and although it is probably in a deprotonated state, it is difficult to imagine a direct effect of this mutation on substrate binding. Taking into account the positioning of substrates into the active site, only the side chains on C6 or C7 of the substrates would be concerned. But then, in the wild-type enzyme, ampicillin and carbenicillin are equivalent as substrates, which would not be the case if they were directly in contact with glutamic acid 104. If new direct interactions were involved, a significant decrease of K_m for cefotaxime with mutant E104K would also be expected. Moreover, substitution by proline has the largest impact on K_m, although it does not have a side chain to interact with the substrate, nor does it modify the conformation of the loop in any visible way. The only antibiotic for which a direct interaction with residue 104 is not excluded is cefaclor. The presence of a chloride on C3 probably induces a different position of this substrate in the active site, in such a way that the ammonium of the R1 side chain of the antibiotic can interact directly with glutamic acid 104. Changing this residue then results in a drastic increase in K_m.

For penicillins, there is no large variations in K_m or k_{cat}. It seems that hydrophobic residues generally have a positive impact on K_m values. Molecular modelling of the various mutations shows that in most cases, there is no steric constraints on the side chain of residue 104, save for tyrosine. In this case, the presence of proline 167, tyrosine 105 and asparagine 132 introduces strong constraints on the conformation of tyrosine 104. The side chain is nonetheless oriented towards the solvent, but stacking-type interactions with the substrates would be possible.

Putting a proline at position 104 does not induce any steric change in the main-chain atoms of the loop, as these atoms are in a favourable conformation. Our modelling shows that it could probably only affect the position of residue 103 side chain. It is noteworthy that this substitution, while abolishing any possibility of hydrogen bonds with the side chain, results in the best K_m and k_{cat} values for almost all substrates. It has to be remembered that residue 104 is involved in hydrogen bonding with residue 132 which is itself involved in substrate binding. In fact, the bottleneck for cephalosporin entry in the active site is between β strand S3 and residue 130, in the 'SDN stretch' between helices H4 and H5. Even a slight displacement of this highly conserved SDN stretch, induced by the abolition of the hydrogen bond between residues 104 and 132 for example could result in a spectacular effect on the entry of cephalosporins in the active site. Such an effect would be analogous, in a much less drastic way, to what Herzberg et al. (1991) have found with mutant P54 of the *Staphylococcus aureus* PC1 enzyme. In this mutant, disordering the Ω loop through a D179N mutation induces a 1.2 Å deviation of alanine 104. In our case, a moderate displacement of the small loop bearing residue 132 could probably explain the relatively important effect of the two substitutions of glutamic acid 104 by proline and tyrosine. This would also be consistent with the fact that mutations at position 104 have little effect if any on the affinity of the enzyme for penicillins. There is by the way a proline at position 104 in the *Streptomyces cacaoi* enzyme, which has rather high K_m values for cephalosporins (Matagne et al., 1990). But as we have already discussed elsewhere (Delaire et al., 1992; Lenfant et al., 1993), contrary to what is generally accepted, the overall sequence dissimilarity between various enzymes within class A often results in different effects for identical residues at the same position, due to small but not negligible differences in the three-dimensional structure.

Thus it seems that the role of residue 104 in defining the substrate range of the TEM enzymes, particularly in the case of the extended-spectrum mutants, is mainly indirect. Changing glutamic acid 104 into a lysine introduces little if any new steric constraints on the enzyme, as the side chain is turned towards the solvent, but it could modify the local electrostatic potential, as well as the geometry of the active site.

It might be wondered why only lysine has been found up to now at position 104 in the natural mutants in clinical isolates. An obvious answer is that this mutation only requires one base change from the glutamic acid codon. In fact, clinical strains are isolated on the basis of a resistant phenotype to third-generation cephalosporins or monobactam, without increase in clavulanic acid resistance. Our results show that although other mutations at position 104 do result in an increased enzymatic efficiency towards these substrates, this only translates in higher MIC values for cefotaxime for the E104K mutant. This is because MIC values reflect a combination of individual factors that can vary for antibiotics even within the same group. For example, cell wall permeability to cefotaxime and ceftazidime are known to be different, as well as the affinity of these compounds for their targets (the penicillin binding proteins) on the cell membrane (Nikaido and Normark, 1987; Sanders, 1992). The β-lactamase, localized in the periplasmic space, acts on the periplasmic pool of antibiotics. Thus, there is probably a threshold effect *in vivo*, reflecting both the high anti-microbial efficacy of these antibiotics and their low periplasmic concentration. This is all the more visible for the clinical isolates, bearing natural plasmids which generally produce much more enzyme that the laboratory constructs we use.

The only natural mutant where mutation E104K is found alone is TEM-17. It probably would have been overlooked were it not for a systematic screening of clinical strains with reduced sensitivity to third-generation cephalosporins (but not true resistance) in synergy with clavulanic acid (Mabilat and Courvalin, 1990). In all the other new TEM enzymes, mutation at position 104 is never found alone but is combined with one or two other mutations (at positions 164, 238 or 264) that contribute significantly to the new hydrolytic capabilities of the enzyme. Like mutation 104, these mutations alone cannot confer a true resistance phenotype to the third-generation cephalosporins in a clinical context. Structural determination of these natural, multiple mutants which is under way will tell us how mutation E104K acts in synergy with mutations G238S, or R164S or H and T264M to result in an extended-spectrum phenotype.

We thank Dr. Alain Reynaud for his gracious help with MIC measurements and Dr. Gilles Moreau for helpful discussions. This work was supported in part by the Ministry of Research and Technology in a joint grant with Roussel–Uclaf (Grant no. 90T0162).

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Received 27 April 1994/13 July 1994; accepted 18 July 1994